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(71) Applicant *(for all designated States except US)*: ZENECA LIMITED [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: JEPSON, Ian [GB/GB]; 31 Gringer Hill, Maidenhead, Berkshire SL6 7LY (GB). GREENLAND, Andrew, James [GB/GB]; Tree Tops, Kingswood Court, Braywick Road, Maidenhead, Berkshire SL65 1DA (GB). BEVAN, Michael [GB/GB]; Cambridge Laboratory, John Innes Centre, Colney Lane, Norwich NR4 7UH (GB). SHEPPARD, Hilary [GB/GB]; Cambridge Laboratory, John Innes Centre, Colney Lane, Norwich NR4 7UH (GB).

(74) Agents: HUSKISSON, Frank, Mackie et al.; Zeneca Agrochemicals, Intellectual Property Dept., Jealott's Hill Research Station, P.O. Box 3538, Bracknell, Berkshire RG42 6YA (GB).

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(57) Abstract

A chemically inducible gene promoter sequence, and particularly, but not exclusively, a chemically inducible gene promoter sequence based on *cis* regulatory elements from the maize glutathione S-transferase 27 (GST-27) gene. In a preferred embodiment, the promoter sequence is operatively linked or fused to a gene or series of genes whereby expression of the gene or series of genes may be controlled by application of an effective exogenous inducer.

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PLANT GLUTATHIONE S-TRANSFERASE PROMOTERS

The present invention relates to a promoter and to a construct comprising the same.

In particular, the present invention relates to a chemically inducible gene promoter sequence, and particularly, but not exclusively, a chemically inducible gene promoter sequence based on *cis* regulatory elements from the maize glutathione S-transferase 27 (GST-27) gene. The present invention also relates to gene constructs, expression systems, plants and promoter/inducer combinations comprising the chemically inducible gene promoter sequence.

Recent advances in molecular biology techniques have resulted in a better understanding of plant promoters. *Cis*-regulatory elements have been identified and used to localise reporter gene activity to specific differentiated cell types and to defined stages of plant development (Drews *et al.*, 1992; Guerrero *et al.*, 1990). While current technology exists to regulate *trans*-gene activity in a spatial or temporal manner, the external control of introduced genes by application of an inducing chemical is not well established in plants.

The ability to regulate genes in an inducible manner is well established in bacteria, fungi, insects and animal cell cultures. For inducible regulation systems to be effective there should be a zero or low level of expression in the absence of inducer, high expression following treatment with inducer and no effect of the inducer on other cellular functions.

Whilst numerous inducible genes have been isolated from plants (Kuhlemeier *et al.*, 1987), a well defined inducible regulation system is not in common use. A number of genes have been described which are activated by pathogen attack or environmental stimuli, including light, oxygen and temperature levels. Although some of these are well characterised at the molecular level, they cannot be utilised for inducible genes system due to illegitimate activation by environmental signals.

The involvement of chemical stimuli, including plant growth regulators, in activation of gene transcription is well documented in plants. Application of these compounds may be better controlled, in comparison with environmental stimuli, however they cannot be considered for inducible genes systems due to undesirable pleiotropic effects.

A number of recent studies have demonstrated that control of *trans*-genes in plants can be achieved by application of exogenous chemicals. These include activation by salicylic acid (Williams *et al.*, 1992), tetracyline (Weinmann *et al.*, 1994], glucocorticoids (Schena *et al.*,

1991) and copper ions (Mett *et al.*, 1993). Although these systems fulfil the prerequisites described earlier, and therefore have utility for research applications, their use will be limited as the chemicals described are not compatible with current agricultural practice.

A potentially attractive group of chemicals which may have utility in regulating gene expression in transgenic plants are herbicide safeners. These compounds are currently used in agriculture and function to selectively elevate the metabolism of certain herbicides, primarily by inducing the detoxifying enzymes, glutathione S-transferase (Hatzios, 1991) and cytochrome p450-dependent mixed function oxidises (Fonne-Pfister and Kreuz, 1990). Glutathione S-transferases (GSTs) are multi-functional enzymes which catalyze the conjugation of the thiol group of glutathione to electrophilic centres of lipophilic compounds leading to their detoxification (Mannervik and Danielson, 1988). GSTs are ubiquitous and their role in xenobiotic metabolism in mammals and plants (Lamoureux and Rusness 1989) is well established.

The best characterised system of plant GSTs is found in maize, where they account for 1-2% of soluble protein (Timmerman, 1989). Four isoforms of GST have been described in maize, GST I (Mozer *et al.*, 1983; Weigand *et al.*, 1986), GST II (Mozer *et al.*, 1983; Holt *et al.*, 1995), GST III (Moore *et al* , 1986) and GST IV (Irzyk and Feurst, 1993). GST-27 is a component of GST II and GST IV which exhibit safener dependent inducibility.

In our International Patent Publication No WO93/01294, the teaching of which is hereby incorporated by reference, we demonstrated that the promoter region controlling GST-27 can be used to achieve safener dependant *trans*-gene expression. These studies revealed that a 3.8 kb GST-27 promoter, in addition to directing safener inducible *trans*-gene expression, also gave a constitutive level of expression in root tissues.

The present invention seeks to provide, by the use of detailed promoter analysis, a deleted GST promoter which still maintains the advantages of chemical inducibility. In this regard, we have, through the use of deletions and mapping of *cis*-regulatory elements, identified sequences involved in safener responsiveness. The use of these sequences can be used to enhance gene switch performance. For example, once a *cis*-element involved in inducible expression has been identified, it is possible to enhance inducibility for example by multimerising the element.

There are examples (copper inducible switch, Mett *et al*, 1993) where inducible promoter systems have been developed for plants where a *cis*-regulatory element conferring inducibility has been fused to a minimal promoter to generate a chimeric promoter responsive to chemical treatment. In this regard, the present invention seeks to provide a chimeric chemically inducible gene promoter sequence comprising a chemically inducible gene promoter sequence of the present invention.

Another aspect of the present invention is therefore to use the *cis*-elements to identify the transcription factors involved in inducible regulation. The transcription factors can then be manipulated to enhance inducibilty, for example a chimeric factor can be engineered with the addition of strong activators, such as the VP16 region from herpes simplex virus.

According to a first aspect of the present invention there is provided a chemically inducible gene promoter sequence comprising 897 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

According to a second aspect of the present invention there is provided a chemically inducible gene promoter sequence comprising 760 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

According to a third aspect of the present invention there is provided a chemically inducible gene promoter sequence comprising 570 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

According to a fourth aspect of the present invention there is provided a chemically inducible gene promoter sequence comprising 378 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

According to a fifth aspect of the present invention there is provided a chemically inducible gene promoter sequence having the sequence of the region 267 to 332 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

According to a sixth aspect of the present invention there is provided a chemically inducible gene promoter sequence having the sequence of the region 275 to 290 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

According to a seventh aspect of the present invention there is provided a chemically inducible gene promoter element having the sequence of the region 267 to 279 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

According to an eighth aspect of the present invention there is provided a chemically inducible gene promoter element having the sequence of the region 278 to 288 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

According to a nineth aspect of the present invention there is provided a chemically inducible gene promoter element having the sequence of the region 286 to 296 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

According to a tenth aspect of the present invention there is provided a chemically inducible gene promoter element having the sequence of the region 320 to 332 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

According to an eleventh aspect of the present invention there is provided a chemically inducible gene promoter sequence or element having substantial homology to the sequences defined above or a variant thereof.

According to a twelfth aspect of the present invention there is provided a DNA molecule comprising one or more of the sequences or elements of the present invention.

According to a thirteenth aspect of the present invention there is provided a multimer comprising more than one copy of any one of the above-defined chemically inducible gene promoter sequences or elements.

According to a fourteenth aspect of the present invention there is provided a chemically switchable gene construct comprising a sequence or element of the present

invention operatively linked to a gene or series of genes whereby expression of the gene or the series of genes may be controlled by application of an effective exogenous inducer.

According to a fifteenth aspect of the present invention there is provided a plant having a construct according to the present invention integrated, preferably stably integrated within its genomic DNA by transformation.

According to a sixteenth aspect of the present invention there is provided a promoter/inducer combination wherein the promoter is the chemically inducible gene promoter sequence or the chemically inducible promoter element of the present invention.

According to a seventeenth aspect of the present invention there is provided an expression system for a plant, the expression system comprising a gene or a series of genes fused to a sequence or element of the present invention wherein the expression system is capable of being expressed in the plant and wherein expression of the gene or series of genes may be controlled by application of an effective exogenous inducer.

According to an eighteenth aspect of the present invention there is provided a transgenic plant comprising a gene construct or an expression system according to the present invention wherein the construct or expression system is integrated, preferably stably integrated, within the plant's genomic DNA.

According to a nineteenth aspect of the present invention there is provided the use of a sequence or element of the present invention to induce expression of a gene or a series of genes, when fused to the sequence or element, in a plant whereby expression of the gene or the series of genes may be controlled by application of an effective exogenous inducer.

According to a twentieth aspect of the present invention there is provided a process of expressing in a plant, a construct or an expression system according to the present invention wherein the expression system or construct is integrated, preferably stably integrated within the plant material's genomic DNA and whereby expression of the gene or series of genes may be controlled by application of an effective exogenous inducer.

The chemically inducible promoter sequence or element is preferably immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase, isoform II or upstream of the 27kD subunit of glutathione S-transferase, isoform IV.

Preferably, the chemically inducible promoter sequence or element is immediately upstream of the transcription start point of the 27kD subunit of maize glutathione S-transferase.

Preferably, the sequence encoding the gene promoter for the 27kD subunit of glutathione S-transferase, isoform II, is as shown in Figure 1.

Preferably, the expression system comprises a gene construct according to the present invention.

A preferred embodiment of the present invention is a chemically inducible gene promoter sequence which is based on *cis* regulatory elements from the maize glutathione S-transferase 27, isoform II (GST-27-II) gene, as shown in Figure 1, or which has substantial homology with that of Figure 1 or a variant thereof , wherein the promoter sequence is operatively linked or fused to a series of genes whereby expression of the gene or series of genes may be controlled by application of an exogenous inducer.

An even more preferred embodiment of the present invention is a chemically inducible gene promoter sequence which is based on *cis* regulatory elements from the maize glutathione S-transferase 27, isoform II (GST-17-II) gene, as shown in Figure 1, or which has substantial homology with that of Figure 1 or a variant thereof , and which is integrated, preferably stably integrated, within a plant material's genomic DNA and wherein expression of a gene or series of genes may be controlled by application of an effective exogenous inducer.

The term "plant material" includes a germinating grain, or a seedling, a plantlet or a plant, or tissues or cells thereof (eg in the root, leaves and stem).

The term "substantial homology" covers homology with respect to at least the essential nucleic acids/nucleic acid residues of the promoter sequence or element providing the homologous sequence or element acts as a chemically inducible promoter. Preferably, there is at least about 70% homology, more preferably at least about 80% homology, and even more preferably there is at least about 90% homology with the chemically inducible promoter sequence or element of the present invention.

The term "a variant thereof" with reference to the present invention means any substitution of, variation of, modification of, replacement of , deletion of or the addition of one or more nucleic acid(s) from or to the promoter sequence providing the resultant

sequence acts as a chemically inducible promoter. The term also includes sequences that can substantially hybridise to the promoter sequence.

The term "construct" - which is synonymous with terms such as "cassette", "hybrid" and "conjugate" - includes a gene or a series of genes directly or indirectly attached to the promoter sequence or element. The same is true for the term "fused" in relation to the present invention which includes direct or indirect attachment.

The term "expression system" means that the system defined above can be expressed in an appropriate organism, tissue, cell or medium. In this regard, the expression system of the present invention may comprise additional components that ensure to increase the expression of the gene or series of genes by use of the chemically inducible gene promoter or element. Also included in this term are transcription factors which are capable of binding to the chemically inducible promoter sequence or element.

The term "transgenic" in relation to the present invention - in particular in relation to the germinating seedlings and plants of the present invention - does not include a wild type promoter in its natural environment in combination with its associated gene or series of genes in its natural environment. Thus, the term includes seedlings or plants incorporating a gene or a series of genes which may be natural or non-natural to the seedling or plant in question operatively linked to the chemically inducible gene promoter sequence or element of the present invention.

The gene switch of the present invention, then, when linked to an exogenous or foreign gene and introduced into a plant by transformation, provides a means for the external regulation of expression of that foreign gene. The method employed for transformation of the plant cells is not especially germane to this invention and any method suitable for the target plant may be employed. Transgenic plants are obtained by regeneration from the transformed cells. Numerous transformation procedures are known from the literature such as agroinfection using *Agrobacterium tumefaciens* or its Ti plasmid, electroporation, microinjection or plants cells and protoplasts, microprojectile transformation, to mention but a few. Reference may be made to the literature for full details of the known methods.

Neither is the plant species into which the chemically inducible sequence is inserted particularly germane to the invention. Dicotyledonous and monocotyledonous plants can be transformed. This invention may be applied to any plant for which transformation techniques

are, or become, available. The present invention can therefore be used to control gene expression in a variety of genetically modified plants, including field crops such as canola, sunflower, tobacco, sugarbeet, and cotton; cereals such as wheat, barley, rice, maize, and sorghum; fruit such as tomatoes, mangoes, peaches, apples, pears, strawberries, bananas and melons; and vegetables such as carrot, lettuce, cabbage, potatoes and onion. The switch is also suitable for use in a variety of tissues, including roots, leaves, stems and reproductive tissues.

One of the main advantages of the present invention as a result of the identification of smaller elements which can be used to achieve inducibility is that the smaller fragments are more convenient for vector development.

Another advantage of defining a core element is that it may be multimerised to enhance safener responsiveness.

A further advantage of using a core element is that it allows safener inducible chimeric promoters to be generated. This may include tissue specific or developmental promoters which could then be manipulated to become safener enhanced.

Another advantage is that such a core element may be optimised via a mutation strategy.

Defining a core element allows the isolation of the corresponding transcription factor by south western screening. This element could then be manipulated by mutation or over expression to achieve enhanced safener dependent expression.

The promoter may be induced by certain chemical compounds such as those shown previously in our International Patent Application No WO90/08826, the disclosure of which is hereby incorporated by reference. known as "herbicide safeners", which can be applied, for example, as a spray, to the growing plant.

The following examples describe the identification of herbicide safener inducible *cis* regulatory elements within the GST-27 promoter. These have been identified by a combination of promoter deletion, *in vivo* footprinting, electrophoretic mobility shift assays, stable and transient reporter gene assays. This has defined specific fragments and a specific core element which have a number of utilities.

Various preferred features and embodiments of the present invention will now be described only by way of non-limiting example with reference to the accompanying Figures in which:-

Figure 1 is a nucleotide sequence of the gene promoter sequence of the 27kD subunit of glutathione S-transferase, isoform II;

Figure 2 is a circular map of plasmid pZM/RMS-3;

Figure 3 is a circular map of plasmid pZM/RMS-3-S;

Figure 4 is a graph showing the inducibility of pZM/RMS-3 and pZM/RMS-3-S in maize;

Figure 5 is a graph showing the effect of treatment with safener on maize stably transformed with RMS-3-S;

Figure 6 shows plasmids pPUG1, pPUG3, pPUG4, pPUG5, pPUG6 and pPUG7;

Figure 7 shows the inducibility of the plasmids pPUG3 to pPUG7, shown in Figure 6;

Figure 8 shows an *in vivo* footprint of the bottom strand of the GST-27 promoter;

Figure 9 shows the maize GST-27 footprint to be complementary to a carnation GST-1 ethylene-responsive element;

Figure 10 shows retard probes for a G290 footprint;

Figure 11 shows an autoradiograph of EMSA using WT290 and MUT290 probes;

Figure 12 shows an autoradiograph of an EMSA competition assay;

Figure 13 shows the results of a transient transformation assay of pPUG5 mutations;

Figure 14 shows partial sequences of retard probes for the GST-27 promoter and ERE_1;

Figure 15 shows retard using WT and MUT probes of G275;

Figure 16 shows retard using WT and MUT probes of G326, G284 and the ERE;

Figure 17 shows the results of a competition assay wherein cold WT probes competed with hot WT290.

Promoter isolation

The isolation and characterisation of the GST-27 promoter region is described in International Patent Publication No WO93/01294. In summary, a 205 bp hybridisation probe, corresponding to the 3' untranslated region of GST-27 cDNA, was amplified using PCR. The PCR product was random primed ^{32}P-labelled and used to screen $5x10^6$ recombinants from a maize genomic library (*Zea mays* cv. W22) in lEMBL3 (Clontech). Plaque purifications were performed as described by Sambrook *et al*, 1989. lEMBL3 DNA was isolated from genomic clones and used for restriction digest mapping and Southern blot analysis. A range of subclones were constructed into plasmid vectors including pG1E7 (3.9 kb *EcoR* I fragment into pBS (Stratagene)), pG1S15 (2.1 kb *Sac* I fragment into pBluescript KS (Stratagene)) and pG1X3 (2.2 kb *Xho* I in pBluesript KS). Plasmid pG1E7 was deposited on 14 June 1991 in the National Collections of Industrial and Marine Bacteria (NCIMB) under accession number NCIMB 40426. The nucleotide sequence of the GST-II-27 promoter is shown in Figure 1.

The DNA sequence of the genomic subclones was determined by the dideoxy chain termination method using sequenase version 2.0 (USB) (Sanger *et al*, 1977). Oligonucleotide sequencing primers were prepared using an ABI DNA synthesiser Model 380B. DNA sequence data was analysed by the use PC/Gene and IG suite of the Intelligenetics molecular biology package.

Testing deletions of the GST-27 promoter in transgenic maize plants

Standard recombinant DNA methods were adopted in the construction of plasmid vectors (Sambrook *et al*, 1989). A reporter gene construct containing a GST-27 3.8 kb *EcoR*I-*Nde* I 5' flanking region from pG1E7 was blunted ended and ligated into the *Sma* I site of the *Agrobacterium* Ti vector pTAK (Jefferson *et al*, 1987). The *Nde* I site, which lies at the predicted translation start codon of *GST*-27 was destroyed after blunting. This formed a convenient point for fusion with the *E.coli UidA* gene encoding b-glucuronidase (GUS) in pTAK. An *EcoR*I fragment containing 3.8kb of the GST promoter GUS reporter gene and the nos terminator was isolated and subcloned in *EcoR*I cut pIJ109, a pUC19 based vector containing the PAT selectable marker cassette (CaMV 35S promoter, AdH I intron, phosphinothricin acetyl transferase (PAT), and the nos terminator), to form the maize transformation cassette pZMRMS3 (see Figure 2). A deletion construct pZMRMS3S was generated containing 0.9kb of GST-27 promoter, by removal of a 1.9kb fragment (*EcoR*I-*Eag*I) (see Figure 3).

pZMRMS3 and pZMRMS3S were used to generate fertile transgenic maize plants by bombardment of embryonic cell suspensions (Fromm *et al*, 1990).

Plants were selected carrying the *trans*-gene using polymerase chain reaction (PCR). Genomic DNA for PCR analysis of transgenic plants was prepared. PCR was performed using the conditions described by Jepson *et al* (1991). Plants transformed with pZMRMS3 and pZMRMS3S were analysed with the primers GSTPCR 5'-CTCCCGTCGACCAAATACACT TGGT-3' specific to the 3' region of GST-27 promoter and GUS 115 5'-GGATTCCGGCATAGTTAAAGAAATCAT-3', specific to the 5' portion of the GUS gene.

To determine if both the 0.9kb and 3.8kb fragments retained inducible expression in transgenic maize, GUS enzyme assays were performed on leaf material in the presence or absence of safener. Induced tissue from mature glasshouse plants (16 H light/8 H dark cycle) was prepared by either leaf paint or spray application of 10g/L R-29148 formulated in 81.5 g/L cyclohexanone, 3.3g/L synperonic NPE 1800, 1.5g/L tween 85. Fluorometric assays for GUS activity were performed with the substrate 4-methylumbelliferyl-D-glucuronide (Sigma) as described by Jefferson *et al*, 1987. Incubations were performed for 2 hours at 37 °C before being stopped with 0.2 M sodium carbonate and fluorescence measured with a Perkin-Elmer LS-35 fluorometer. Protein concentration of tissue homogenates were determined by the Bio-

Rad protein assay following the manufacturer's suggested procedure. Figures 4 and 5 demonstrate that both pZMRMS3 and pZMRMS3S retain inducibility.

Fine deletion of the GST-27 promoter

The preliminary deletion analysis generated in transgenic maize plants suggests that the element/s conferring inducibility must lie within the 900bp immediately upstream of the transcription start point (TSP). A series of fine deletion constructs were made by fusing 200bp deleted fragments of the 900bp region to GUS marker gene. A *Pst* I site was identified adjacent to the transcription start point of the GST-27 promoter. A PCR primer AI2 was designed corresponding to this region (5' TGCCTGCTGCAGCTGCTACTTAT 3'). Primer AI2 was used in combination with 4 primers (AI3, AI4, AI5 and AI6)) all flanked with a *Hind* III site. AI3 (5' GTTAAAGCTTCGCAAGTCGCACCCCACTA 3'), AI4 (5' CTGAAAGCTTCGGTGCACCGAAT 3') , AI5 (5' GCGGCAAGCTTAATATGTG ATGATGATA 3') and PI6 (5' TTACAAGCTTCGCAAGTATCGGTAGGCAT 3') were used in PCR experiments with AI3 to generate fragments of 217bp, 378bp, 570bp and 760bp respectfully. The PCR products produced were cut with *Pst* I and *Hind* III and the fragments cloned into pZMRMS3 cut with *Pst I* and *Hind* III. The resultant vectors pUG4, pUG5, pPUG6 and pPUG7 are shown in Figure 6.

Transient transformation assays were performed with the pPUG vectors in BMS (Black Mexican Sweet) suspension cells, grown in the presence or absence of safener (dichlormid 40ppm). DNA was delivered using silicon carbide whisker transformation (Wang *et al*, 1994). Promoter activity was scored by counting colour forming units. Figure 7 reveals all the pPUG constructs were inducible, except for one containing only 217bp (designated pPUG7) of the promoter. A construct containing 378bp (designated pPUG6) still conferred inducibility. This data suggests that the inducible element/s lay between -217 and -378 bp upstream of the transcription start point.

Mapping inducible elements within the GST-27 promoter using *in-vivo* footprinting

In vivo footprinting (see method below) was used to detect proteins interactions with the promoter and so locate the element which confers inducibility. Dimethyl sulphate (DMS) is used to modify guanine residues *in-vivo* by methylating the N7 position. If a protein is

closely associated with the DNA this reaction will be inhibited and so DNA-binding factors can be mapped to the bases involved. After DMS treatment the DNA is amplified and sequenced. Contact points are identified on genomic autoradiographs as G residues that are less intense when compared to the lane of uninduced DNA.

Primers were designed so that the area between -217 and -378 could be analysed using this method. A maize plant was treated with safener to induce expression of GST-27. *In-vivo* footprint analysis was performed before the treatment (0 hours) and at 6, 24 and 48 hours after treatment. Results are shown in Figure 8. It can be seen that a protein binds to a G residue at position -290 at 24 hours after the plant has been treated with safener (no band visible) but not at 0,6 or 48 hours (band visible). Positions -275, -283 and -284 also have fainter bands at 24 hours. This result is reproducible. In short we have identified 2 putative elements which appears to bind protein factors in a safener dependent manner.

These elements share homology with each other. In addition it is interesting to note that the homologous regions are complementary to a known ethylene responsive element in the GST1 gene in carnation (Itzhaki *et al*, Proc. Natl. Acad. Sci. USA Vol. 19, 8925-8929, 1994) as shown in Figure 9.

Protocol for *In-vivo* Footprinting of Maize leaves:

(Modified from Hammond-Kosack and Bevan, Plant Mol. Biol. Reporter, Vol. 11, No. 3, Sept. 1993)

1. *In-vivo* DMS treatments applied for 1 minute and then slowly released. After 5 minutes the medium was removed and the tissue was washed several times in MS media. The tissue was blotted dry and stored at -70°C until all time points (0, 6, 24 and 48) hours were collected.

2. Preparation of chromosomal DNA:

The frozen DMS treated tissue was crushed to a fine powder using a pestle and mortar and liquid nitrogen. 30ml hot (65°C) extraction buffer (100mM Tris HCl pH 8.0, 50mM EDTA pH 8.0, 500mM NaCl, 1.25% SDS, 8.3mM NaOH, 0.38g/100ml Na bisulphite) was added, mixed and incubated for 15 minutes at 65°C. 6.16ml 5M KAc were added and then the sample was incubated on ice for 20 minutes. After centrifugation at 3.5K for 5 minutes the

supernatant was filtered through Miracloth and 0.7 vols propan-2-ol were added. This was spun at 4K for 10 minutes, the pellet was washed twice in 70% ethanol and then resuspended in 0.84ml T5E and 0.36ml 10M NH4Ac. This solution was spun for 5 minutes at 13K. The supernatant was precipitated with 0.73ml propan-2-ol.

The pellet was precipitated once more before dissolving in 100ml T10E. The DNA was digested with *Hind* III.

3. Ligation-mediated PCR (LMPCR):

The DNA samples were amplified using LMPCR. 3 nested primers were designed to amplify both strands of the GST-27 promoter between 0 and 350bp upstream of the TSP. The 1st primer for each strand was annealed and then extension to the end of the molecule was allowed to occur. A linker of known sequence was annealed to the end of each extended molecule. Normal PCR was then carried out using a primer specific for the linker and the 2nd of the nested primers. When amplified the DNA molecules were labelled using the 3rd primer incorporating an end-label. The samples were phenol-chloroform extracted, propan-2-ol precipitated and resuspended on sequencing loading buffer. To visualise the footprint the samples were run in a 6% sequencing gel.

Electrophoretic mobility shift assays (EMSA):

This method was used to test the hypothesis that the *in-vivo* footprinted areas could bind specific nuclear proteins *in-vitro*. Short radiolabelled fragments (probes) of a promoter will migrate through an electrophoresis gel at a speed determined by their size and charge. If a probe has a protein associated with it the migration will be retarded. On an autoradiograph this will be visualised as a band which is not present in the absence of protein. Competition assays with cold probes determine if binding is specific.

Nuclear protein extracts were made from induced and non-induced maize leaves. Two short (25bp) probes (see Figure 10) were made to incubate with the protein:

1. WT290 - covering the -290 and -283/284 footprinted area
2. MUT290 - as 1 but including a 5bp mutation around -290.

Results are shown in Figure 11. The WT probe binds a protein (band visible in lanes 3 and 5). The mutation in MUT290 abolishes binding (weaker band in lanes 4 and 6). The pattern is identical whether the nuclear protein extract is from induced or non-induced leaves. Therefore the binding protein must always be present. *In-vivo* this protein must be modified such that binding only occurs when the gene is induced. Fig. 12 represents the results of competition assays. Cold WT290 probe successfully competes with radiolabelled WT290 probe to bind the protein resulting in loss of the band (lanes 2, 3, 4 and 10, 11, 12). This effect is not seen when cold MUT290 is added (lanes 6, 7, 8 and 14, 15, 16). Therefore the binding seen is specific.

In addition to the 290 region, it has also been demonstrated that the -275 region (see Figure 14), is involved in transcription factor binding. Figure 15 shows the results of an EMSA in which the 275 probe binds while the mutated 275 element fails to bind transcription factors. In a subsequent experiment the 284 and 326 (see figure 14) regions have been shown to be involved with binding of protein from uninduced and induced nuclear extracts (see Figure 16). Competition assays (see Figure 17) with the 275, 284, 290, and 326 regions show that the binding observed in the EMSA study is specific. The various mutated and wild probes used in the EMSA study are listed below:

Probe name	Sequence
290WTN	5 AGCTT GC TATTTCAGAAT GC A3
	3 A CG ATAAAGTCTTC AG TTCGA 5
290MUTN5	5 AGCTT GC TATGGCCTAAT GC A3
	3 A CG ATACCTGATTA CG TTCGA 5
284WTN	5 AGCTT GC GAATCCGAAAT GC A3
	3 A CG CTTAGGCTTTA CG TTCGA 5
284MUTN6	5 AGCTT GC GACGAATCAAT GC A3
	3 A CG CTGCTTAGTTA CG TTCGA 5
275WTN	5 AGCTT GC AATTTCATAAA GC A3
	3 A CG TTAAAGTATTT CG TTCGA 5
275MUTN5	5 AGCTT GC AATGGACGAAA GC A3
	3 A CG TTACCTGCTTT CG A 5
326WTN	5 AGCTT GC GGTTCCTAAAA GC A3

	3 A CG CCAAGGATTTT CG TTCGA 5
326MUTN6	5 AGCTT GC GGCGGAAGCAAAGC A3
	3 A CG CCGCTTCGTTT CG TTCGA 5
ERE-WT	5 AGCTT GC TATTTCAAAAT GC A3
	3 A CG ATAAAGTTTTA CG TTCGA 5
ERE-MUT	5 AGCTT GC TATGGTCCAAT GC A3
	3 A CG ATACCAGGTTA CG TTCGA 5

These data taken together with the in vivo footprinting data indicate that this region of the GST-27 promoter is involved in chemical dependent binding of transcription factors leading to gene activation.

Protocol for Electophoretic Mobility Shift Assays (EMSAs):
(modified from Watson and Thompson, Meth. Enzymol. Vol. 118, pp. 57-75, 1986 and Holdsworth and Laties, Planta, Vol. 179, pp. 17-23, 1989)

1. Preparation of nuclear protein extracts from maize leaves:

A 1% solution of R-29148 safener was applied to the upper and lower surfaces of 3 week old maize leaves. 15g of material was harvested at 0 and 24 hours. The tissue was crushed to a fine powder using a pestle and mortar and liquid nitrogen. The tissue was divided into 2; to each sample 70 ml extract buffer (0.25M sucrose, 10mM NaCl, 10mM MES-NaOH pH 6.0, 5mM EDTA, 0.15mM spermine, 0.5mM spermidine, 0.2mM PMSF, 10mM NaF, 20mM b-ME, 0.1% BSA, 0.6% non-idet P40) was added. The homogenate was filtered through 3 layers Miracloth to a corex tube. A 25% Percoll cushion was added to the bottom of the tube. This was spun at 3K in a swing bucket rotor at 4°C for 30 minutes. Nuclei were collected from the bottom of the tube and resuspended in extract buffer (not including BSA). The sample was spun at 5K for 5 minutes at 4°C. The nuclei were resuspended in 100ml dialysis buffer (40 mM KCl, 24.7 mM HEPES-NaOH pH 7.9, 5mg/ml leupeptin, 5mM EDTA, 5mg/ml antipain, 1mM DTT, 25% glycerol, 10mM NaF). A 1/10th volume of ammonium sulphate was added and the sample incubated on ice for 30 minutes, then spun at 13K for 20 minutes. 1.5 volumes of ammonium sulphate were added to the supernatant which was incubated on ice for 60 mins and then spun at 13K for 20 minutes. The sample was

resuspended in 100ml dialysis buffer and dialyed in the buffer for 2 X 2 hours. The protein concentration in the final sample was measured using Bradford's assay.

2. EMSA:

1mg protein extract was incubated for 20 minutes at room temperature with 20000cpm (c. 0.5ng) probe (labelled by filling in the 3' recessed termini with labelled dNTPs using Klenow), 1mg poly d(I-C), 5mM DTT, 50 mM KC1, 100mM $MgCl_2$ in 10ml binding buffer (250mM HEPES pH7.6, 10mM EDTA, 50% glycerol). The samples were loaded on a 6% olyacrylamide gel run in 0.25X TBE buffer.

Transient expression assays

To test that the footprinted areas indicate the positions of inducible elements transient assays were performed. By mutating the putative inducible elements to prevent *trans*-acting protein factors binding, inducibility should be lost. A construct (pPUG5) containing 570bp of the promoter fused to GUS was previously shown to retain inducibility. This was mutated, using anchored PCR, in the areas that had been footprinted. Three constructs were made, each containing a 10bp mutation:

1. MUT290 containing a 10bp mutation around G-290
2. MUT284 containing a 10bp mutation around G-284
3. MUT326 containing a 10bp mutation around G-326

The MUT326 construct was made as a convincing footprint was seen at 48 hours involving G-325/326. The constructs were transformed into BMS suspension cells using the silicon fibre so-called whisker technique as described in our US Patent No 5,302,523. Results are shown in Table 1 (Figure 13). The mutated constructs were still inducible. The fold induction for all constructs was comparable to that of WT pPUG5.

Following this result a homology search was performed on the 570bp fragment in pPUG5 to identify any duplication of the putative elements. If multiple elements are present the effect of mutating one may not be visible. No direct duplications were found. However,

it was observed that there was high (67%) homology within the fragment. From the data it is believed that a duplication and inversion event involving 150bp had occurred. In addition, as the -290 and -275 footprinted region show significant homology the transient assay experiment was repeated in the pPUG6 (373bp) backround promoter, with all three mutaions in the same vector.

Plant transformation vectors

PCR-mediated mutagenesis of GST-27 promoter

Four primers were required for PCR-mediated mutagenesis, the primers used are listed below:-

Primers used to create 3XMUT in pPUG6:

R1 = 5' CTGAAAGCTTCGGTGCACCGAAT 3' (pAI4)

R2 = 3' TATTCATCGTCGACGTCGTCCGT 5' (pAI2)

M=5'CCTAAAATTATTTTAAAAATTTTGGTTCTCATATGGACTACGAATCAA
TGGACGAAATCCAAATAGACCG 3'

rev = 3' GGATTTTAATAAAATTTTTAAAACCAAGAGT 5'

Primers R1 and R2 flank the promoter on either side of the region which is to be mutated and contain suitable enzyme restriction sites for cloning. The 5' and 3' ends of primer M are identical to the promoter while the middle section contains the altered sequence. Primer rev is identical to the wild type 5' end of primer M.

In step 1, two PCRs were performed in a volume of 25 µl with 20 ng wild type template plasmid, 10X PCR buffer, 2.5 µl 2mM dNTPs, 2 µl 20 mM primer R1 or M, 2µl 20 mM primer rev or R2 and 0.25 µl Amplitaq (Perkin Elmer). The program used was 94^{0}C for 2.5 minutes followed by 30 cycles of 94^{0}C for 1 minute, 50^0 C for 1 minute and 72^0 C for 1 minute. The reactions were each carried out 4 times to generate enough product for step 2. The resulting fragments R1/rev and M/R2 were purified and quantified.

In step 2, the fragments R1/rev and M/R2, which share a region of overlapping sequence, were annealed. 250 ng of each fragment were mixed in a 25µl volume with 10X PCR buffer, 2.5µl 2 mM dNTPs and 0.25µl Amplitaq. Annealing and then extension of the

annealed product were performed using the program 94°C for 2.5 minutes followed by 9 cycles of 94°C for 1 minute, 40°C for 1 minute and 72°C for 1 minute.

In step 3, the extended heterodimers, comprising of the whole promoter sequence containing the mutation, were amplified by the addition of primers R1 and R2. To the same reaction from step 2, 2.5 µl 10X PCR buffer, 2.5 µl 2 mM dNTPs, 2 ml 20 mM R1, 2 ml 20 mM R2, 15.75 µl of water and 0.25 µl Amplitaq were added. PCR was carried out using the same program as that which was used in step 1. The resulting fragment was purified and cloned into pGEM-T vector (Promgea) and sequenced to ensure that the correct bases were mutated. 3XMUTBin/6 contained 5 bp mutations to the footprinted G residues G-290, G-283/284 and G-275 and their flanking nucleotides.

A similar strategy was adopted to generate 4 mutations in the pPUG6 background using the primers given below.

Primers used to create 4XMUT in pPUG6:

R1 = 5' CTGAAAGCTTCGGTGCACCGAAT 3' (pAI4)

R2 = 3' TATTCATCGTCGACGTCGTCCGT 5' (pAI2)

M =5' GTCTATTCAGGTTCGGGGAAGCAAATTAT 3'

rev = 3' CAGATAAGTCCAAG 5'

Further PCR mediated mutagenesis was performed using p3XMUT/PUG6 as template DNA in order to mutate the footprinted G residues G-325/236 and four flanking nucleotides. The 4XMUTBin/6 contained 5 bp mutations to the footprinted G residues G-290, G-283/284, G-275 and G-325/326 and their flanking nucleotides.

The mutated fragments were introduced into the GST-27 promoter by cutting the pGEM-T vectors with HindIII and PstI and cloned into pPUG6 which had been linearised with the same enzymes. In this way the wild type promoter in pPUG6 was removed and replaced with the 3XMUT or 4XMUT version, so creating the plasmid p3XMUT/PUG6 or p4XMUT/PUG6. This was cloned into pBin400 using the same strategy as described above for the wild type pPUG6 plasmid, to form 3XMUT/Bin6 or 4XMUT/Bin6.

Vector construction

Tobacco plants were transformed with five constructs, all of which were based on the binary vector pBin400 (Spychalla and Bevan, 1993). Three 5' deletions of the GST-27 promoter fused to the reporter gene GUS were prepared, containing 570, 378 and 217 bp of the promoter upstream from the TSP. These truncated promoter:GUS fusions were present in the constructs pPUG5 (570 bp), pPUG6 (378 bp) and pPUG7 (217 bp). The truncated promoters fused to GUS were cut out on a HindIII, EcoRI fragment from these three plasmids and cloned directly into pBin400, which had been linearised with the same enzymes. The resulting constructs were named pBin/5, pBin/6 and pBin/7. Also transformed were 3XMUT/Bin6 and 4XMUT/Bin6.

Transformation of *Agrobacterium*

An overnight culture of *A.tumefaciens* (strain T37SE) was set up in 10 mls YEP broth (1 % (w/v) Bactopeptone [Difco], 1 % (w/v) yeast extract [Difco], 0.5 % NaCl) with 50 µg/ml kanamycin sulphate and grown at 30 °C. Four mls of this were used to inoculate 100 mls YEP broth, containing 50µg/ml kanamycin sulphate, which was grown for 4-5 hours. The cells were pelleted at 2,500 rpm for 10 minutes (Sorvall RC3C centrifuge, H6000A rotor), resuspended in 2 mls YEP broth and chilled on ice for 5 minutes in 200 µl aliquots. Ten microlitres pBin DNA at 0.1-0.2 µg/ml were added to the cells. The cells were immediately frozen in liquid nitrogen for about 15 seconds followed by a heat shock of 37° C for 5 minutes. 1 ml YEP was then added and the cells grown at 30° C for 1-2 hours. A 100 ml aliquot from each transformation was spread onto YEP plates (YEP with 1.5 % (w/v) agar) containing 50µg/ml kanamycin sulphate to select for the *Agrobacterium* and 50µg/ml spectinomycin hydrochloride to select for the binary vector. The plates were inspected for growth after 48 hours incubation at 30 °C. Single colonies were streaked out on Minimal T plates (350 mls water agar [1.5 % (w/v) agar], 20 mls 20X salts, 20 mls 20X T buffer, 20 % glucose), containing the selecting antibiotics, and grown for 3 days at 30° C. Bacterial colonies from these plates were used to inoculate liquid cultures for DNA preparation.

Plasmid DNA preps from *Agrobacterium, tumefaciens*

Ten mls YEP broth, containing antibiotic to select for the binary vector and the *Agrobacterium*, were inoculated with a single colony of *A. tumefaciens* and grown overnight at 30° C. The cells were collected by centrifugation at 2,500 rpm for 20 minutes (Sorvall RC3C centrifuge, H6000A rotor), then resuspended in 200 ml ice-cold solution I (Horsch et al 1985) and allowed to stand for 30 minutes at room temperature and then vortexed. 200 ml solution II were added, mixed by gentle shaking and incubated at room temperature for 30 minutes. 150 ml ice-cold solution III were then added and the mixture was incubated on ice for 5 minutes. The sample was centrifuged for 5 minutes at 13,000 rpm (MSE Microcentaur benchtop microfuge). The supernatant was extracted with an equal volume of phenol/chloroform and then IPA precipitated. The pellet was resuspended in 50 ml T10E1. RNA was digested by the addition of 1 ml 1mg/ml RNaseA, followed by incubation at 37° C for 30 minutes. Ten microlitres of this DNA were used in restriction enzyme digests and the identity of the binary plasmid was confirmed by agarose gel electrophoresis.

Transformation of tobacco

Tobacco was transformed using the method developed by Horsch et al.(1985). The *Agrobacterium* containing the binary vector construct was grown in YEP broth, containing the appropriate antibiotics, at 30°C for 24 hours prior to the transformation. The cells were pelleted by centrifugation at 3,000 rpm for 20 minutes at room temperature (Sorvall RC3C centrifuge, H6000A rotor) and resuspended in 10 mls fresh YEP. Leaf discs were cut from leaves of *Nicotiana tabacum* var. Samsun using a cork-borer (7mm) and transferred to a 14 cm petri dish. The *Agrobacterium* was added to the petri dish which was left at room temperature for 30 minutes. Feeder plates were prepared before starting the experiment. One l NBM media (4.6 g MS salts [Flow Laboratories], 30 g sucrose, 0.1 mg EtOH NAA [Sigma], 0.1 mg BAP [Sigma], 8 g agar [Difco], 100X B5 VITS, pH 5.9) was required for 40 plates. One ml of a tobacco cell suspension culture was spread evenly over the surface of the plates, and then covered with a sterile 9 cm Whatman No. 1 filter disc. The leaf discs were transformed to the feeder plates, lower epidermis uppermost. After 2 days co-cultivation with the *Agrobacterium* the discs were transferred to NMB plates containing 100 µg/ml kanamycin sulphate and 500 µg/ml carbenicillin sodium salt, in order to eliminate the Agrobacterium and

select for transformants. The leaf discs were transferred every 2 weeks to fresh NMB plates containing the antibiotics. After approximately 4 weeks small shoots were visible on the leaf discs. These were transferred to MS medium (4.6 g MS salts, 30 g sucrose, 8 g agar, pH 5.9) containing 200 µg/ml carbenicillin sodium salt and 100 µg/ml kanamycin sulphate. Transformants rooted after approximately 14 days while untransformed plants bleached and died. When the transformed plants were established they were transferred to soil and grown in the greenhouse.

Other modifications of the present invention will be apparent to those skilled in the art without departing from the scope of the invention.

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CLAIMS

1. A chemically inducible gene promoter sequence comprising 897 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

2. A chemically inducible gene promoter sequence comprising 760 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

3. A chemically inducible gene promoter sequence comprising 570 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

4. A chemically inducible gene promoter sequence comprising 378 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

5. A chemically inducible gene promoter sequence having the sequence of the region 267 to 332 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

6. A chemically inducible gene promoter sequence having the sequence of the region 275 to 290 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

7. A chemically inducible gene promoter element having the sequence of the region 267 to 279 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

8. A chemically inducible gene promoter element having the sequence of the region 278 to 288 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

9. A chemically inducible gene promoter element having the sequence of the region 286 to 296 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

10. A chemically inducible gene promoter element having the sequence of the region 320 to 332 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

11. A chemically inducible gene promoter sequence or element according to any one of the preceding claims wherein the sequence or element is immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of maize glutathione S-transferase.

12. A chemically inducible gene promoter sequence or element according to any one of the preceding claims wherein the gene promoter sequence of the 27kD subunit of glutathione S-transferase, isoform II, is as shown in Figure 1.

13. A chemically inducible gene promoter sequence or element having substantial homology to the sequences or elements of any one of claims 1 to 12, or a variant thereof.

14. A DNA molecule comprising one or more of the sequences or elements of any one of claims 1 to 13.

15. A multimer of the sequences or elements of any one of claims 8 to 13.

16. A gene construct comprising a sequence or elements of any one of claims 1 to 15 operatively linked to a gene or series of genes whereby expression of the gene or the series of genes may be controlled by application of an effective exogenous inducer.

17. A plant having a construct as claimed in claim 16 integrated, preferably stably integrated within its genomic DNA by transformation.

18. A promoter/inducer combination wherein the promoter is the chemically inducible gene promoter sequence as claimed in any one of claims 1 to 6 and claims 11 to 13, when dependent on claims 1 to 6, or the chemically inducible promoter element of any one of claims 7 to 10 and claims 11 to 13, when dependent on claims 7 to 10.

19. An expression system for a plant, the expression system comprising a gene or a series of genes fused to a sequence or element as claimed in any one of claims 1 to 15 wherein the expression system is capable of being expressed in the plant and wherein expression of the gene or series of genes may be controlled by application of an effective exogenous inducer.

20. An expression system according to claim 19 comprising a construct according to claim 16.

21. A transgenic plant comprising a gene construct according to claim 16 or an expression system according to claim 19 or claim 20 wherein the construct or expression system is integrated, preferably stably integrated, within the plant's genomic DNA.

22. The use of a sequence or element as claimed in any one of claims 1 to 15 to induce expression of a gene or a series of genes, when fused to the sequence or element, in a plant whereby expression of the gene or the series of genes may be controlled by application of an effective exogenous inducer.

23. A process of expressing in a plant, a construct according to claim 16 or an expression system according to claim 19 or claim 20 wherein the expression system or construct is integrated, preferably stably integrated within the plant material's genomic DNA and whereby expression of the gene or series of genes may be controlled by application of an effective exogenous inducer.

24. A promoter, a construct or an expression system substantially as hereinbefore described with reference to any one of Figures 1, 2, 3, 6 or 14.

Fig.1.

gaattccaaatatatgatgattgttgtcctagtgcagaagaactaaatatactagcgaaaaaaaccttc
ctagtcatgtaagtgtatgggcatatagaaaaataaacatctcaagactccaaactagtcatagctttta
gtcacaacttcaaacacttcatgccaacaagatcatggatttttttttttgcctaagacaaaactagaat
gagaaaagaactaactcatcatacatattagtatggcatcacaaaaaaaatgacacatatatgatACTAT
ATCACACAGGCCTTCAGTTTCTAGAACAAGTGCAGATCGAtgtgtgggtatgcatgtctaatattttact
aggttggatatgcatgggcgttcattcagaatcagtttcacacagtttatcgcacttctgtttacaaaac
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caggaaactgatgtgcaacaacAAAAACTAAAATAGGAAGGaaacacaagttccaaatgtataataattg
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GCAAACTAGTCACAACTTTAAACATTTCATGCCAACAAGATCATGGATGGTGTTTTTTTTTCCTAGGGAA
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CTATATCACGCAGCCGTTCAATTTCTAGAACAAATGCAGATTGATCTGTGAATATGCATGTCTCATATTT
TACTAGGTTGGATGGACTGAATCCCGTGAAACAAACAATTTATTCAACAAgtttctgcatgaatatcatc
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attgtttacccatcaatttcaagtacacagtaacaagaacagtacagctagaattgagcatgtgagtATT
GTTGATACCTCGTTGAGCTCTCTCTGCCGCGGCTTTCTGCTCGGCagcaagagccagctcaggatccacc
ccgaaagcttgggcgtaggtgttgtctatcggcgaaaacacgcgcggtacgccaagaacagcgcggccat

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Fig.1(Cont i).

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ctccatcccaggcacggtgcgcccgcttttcgccgtctcgctgagtcacggcgggcgtccagcaggtag
ttgagcgccttccgcggcacgaatcgctgcgtgcggcccGGATCTGGTCGAGTTGGTAGTCAGCGTCGGT
GTCGAATGCCGGGACGTCgaccaggaagaagttgccgtcgctggggtggggacggaaggcgtcaggattg
tcgcaagggcagagcccagcCtGCGGgcgGGGCTACCTCGTCGACGCCTCGgCACGGCGGCGgCAAAGCT
GcTGCGGGAcgTGCccgCCTGGGCCGCCTTCTCGGTGAAGTGGtCCtcgaaggggacgagctcgctgggg
tcaaaccacccccatagctcGAGTCACcGAAgAAGGCGACGAGGACGAGCCCGTcgcGGTGGCCgcgGTGT
ACCTCCTCGTCGTCGGTGAggctGACGCTGTAGATATGGCCAGGCcACCACGGATGGGACTTCACCTTGG
CCCAGACCATGTcGcCGAACCGGGGGCCGCcGTTCGCCCATGCGATgCcGCGTCCGGCAGCAGGAACCAT
GGcGCCTCCAGCGGCGGGGTCGGACATCCTGTGGAGGGGAACCGAAAACCTAGATTTGGATGCAGGTTCG
ATTGGTCTGGGCTTGGGTTTGGgTTCCGGAGgAGGGTGGCCTGGGATCGGTGGAAGGAGGGACATTGTTG
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CACATTCTCTATTTTAAATTTCACTTTGTGTAATCTACACtATAAAATAGTGTTTTACACGGTATGTTGT
ACACAGCCTTATCGTGgCGCGAcgGAGTTGGATAGAGATGGTGAACAGCTGGATAGATATGATTTATAGG
CGATTGGGTAGATGTGATTTGATAGGTGGTTATGTAGGAGCGATTTAGTGAGACATTGTAAATAATTAGG
TTGATGTGATCCGAGGATGGCTAGGTAGATATGATTTTAATGGATGGTTTGGTGGACTAAGTTATGTGGA
CATTATAATATGTTTTAAATTTCTAAGAAATTGTTTGTGTTAAATTGTATCCCACATAGATTATTTAGCC
ATCTCAAAGAGAGGTTTGGGTTGTTTACACAAATAAAATATTCGTTTGCTTCTACAATTTATATGTTTTT
TATTTACATGAAAACTATATTTTTTATTCATCTACTCACCCAGCACAGAAATTCTGGTTGAGTAGATGAA
```

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Fig.1(Cont ii).

AAAAAACTACAACAAACTCTTCCTGAAAGTGTCGGTGTGAAGCCGAGAAATCCTTTTCATTTCGGTGACG
GAGCCCCTTGCTGGCTGCTGCTCAGTGCACTCCGTTCGCCTGCCTGCCACTACAAGCGACGGCCGACGAC
TCGCAAGTATCGGTAGGCATTTTAAAACTGAAAACCAAATCTAAACCCGAATAGACCAAATTGTTGGTTT
ATTCGGGTTTTTGGGTTCGGATTCGGTTTCTAAATATGCTATATTTTAGGGTATAGGTTCGGGTTCAGTT
TCTAACCTTTAAAACCTGAATAGACGAATAACCCGAAATATAAAAAATCTCTTAATATGTGATGATATTA
TTATATGATTTATGAACTTATTAACCGAAAATAATGATACCATCCTAACGATAGTATATATATCTATGTA
TGCTATTTTTATAGTCACTTGTTGTAATAATAGTACTTCCAATTAATTAATCAGTGTATATATTTTAACA
AAAGATACTAGCcTCTCTACTATTTGAGTATATTCGGTGCACCGAATAGACCGAACCGAAATTGTAAGTC
TATTCAGGTTCGGTTCCTAAAATTATTTTAAAAATTTTGGTTCTCATATTTCAGAATCCGAAATTTCATA
AATCCAAATAGACCGAACCAAATTaCGCTAATAGACCGAATAACTAGCGTACTCgCAAGTcGCACCCCAC
TAGCCTGCTGCGTGCGTAAGCGAGGACGTCACGCGTTCTCCCTCCCGTCGACCAAATACACTTGGTCTTC
TAGCACCTTCTTCCTCTCCAAGaCTCCAATCCCCCAACCACCAGAACCAGCGCCagCTctAACGTCACCT
CTGATTTCTCTCTCCTCTCTATTGCTAGCTGCTTTATTATAAGTAGCAGCTGCAGCAGGCAGGAGCTGCA
CACACCCATCCAATTCCAGCTGCTGATCTTGATCCTGCACCCCGAGCCGTACACAAGAGCTAGTCGGTAG
AAcTTGCAGGAGCGGAgcAGAACTAAGTGCAGAGAACAGGACATATG

TSP

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Fig.2.

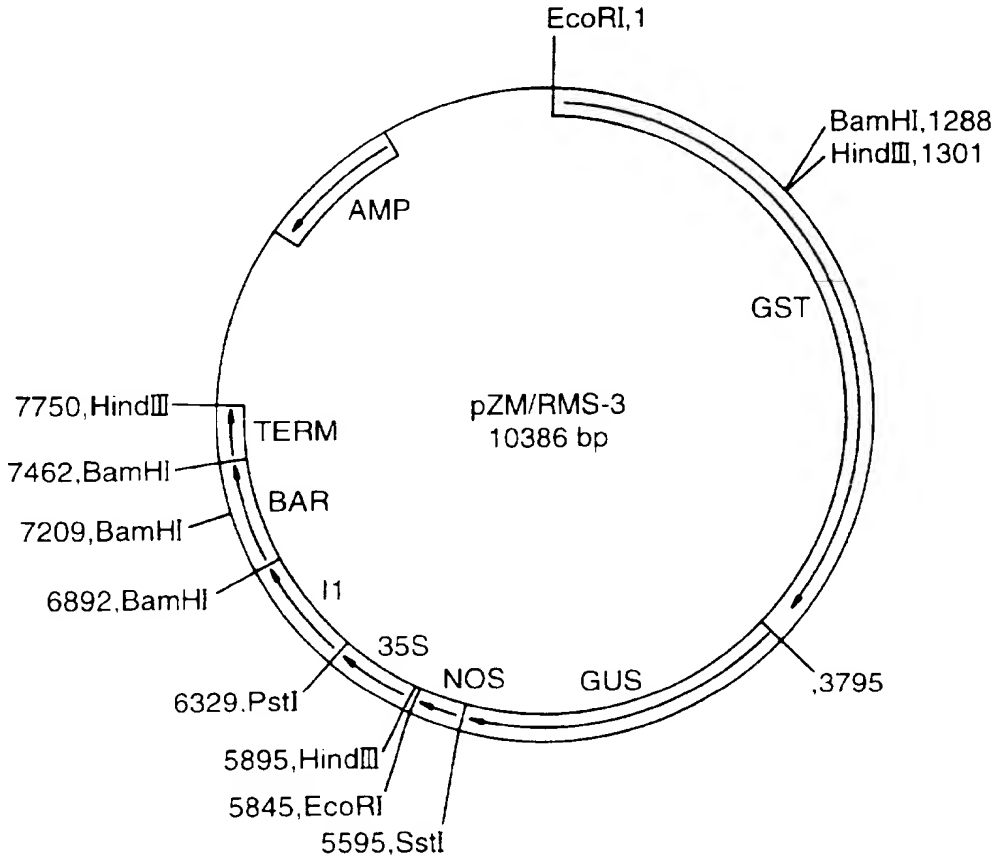
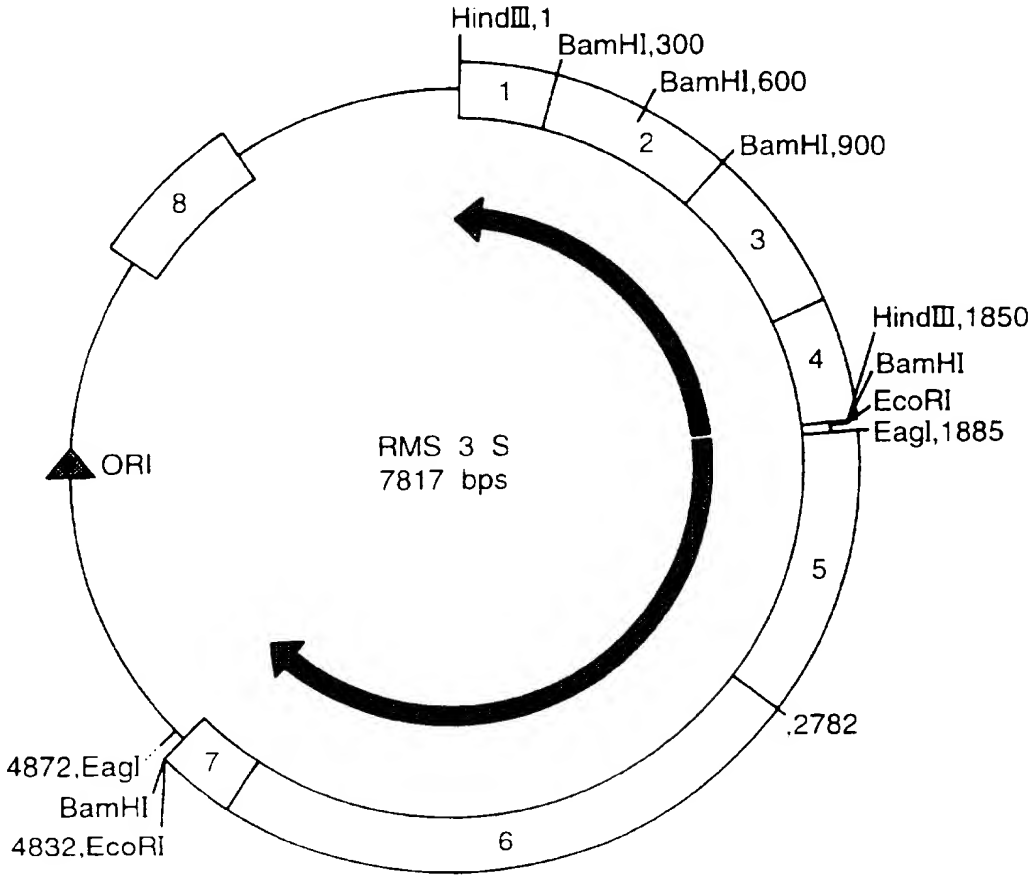

EcoRI,1
BamHI,1288
HindIII,1301
AMP
GST
pZM/RMS-3
10386 bp
7750,HindIII
TERM
7462,BamHI
BAR
7209,BamHI
6892,BamHI
I1
35S
NOS
GUS
,3795
6329,PstI
5895,HindIII
5845,EcoRI
5595,SstI

Fig.3.



KEY TO GENES

1=nos TERMINATOR
2=BAR GENE
3=Adh 1 INTRON
4=CaMV 35S
5=GST PROMOTER
6=GUS GENE
7=nos TERMINATOR
8=AMPICILLIN RESISTANCE

Fig.4.

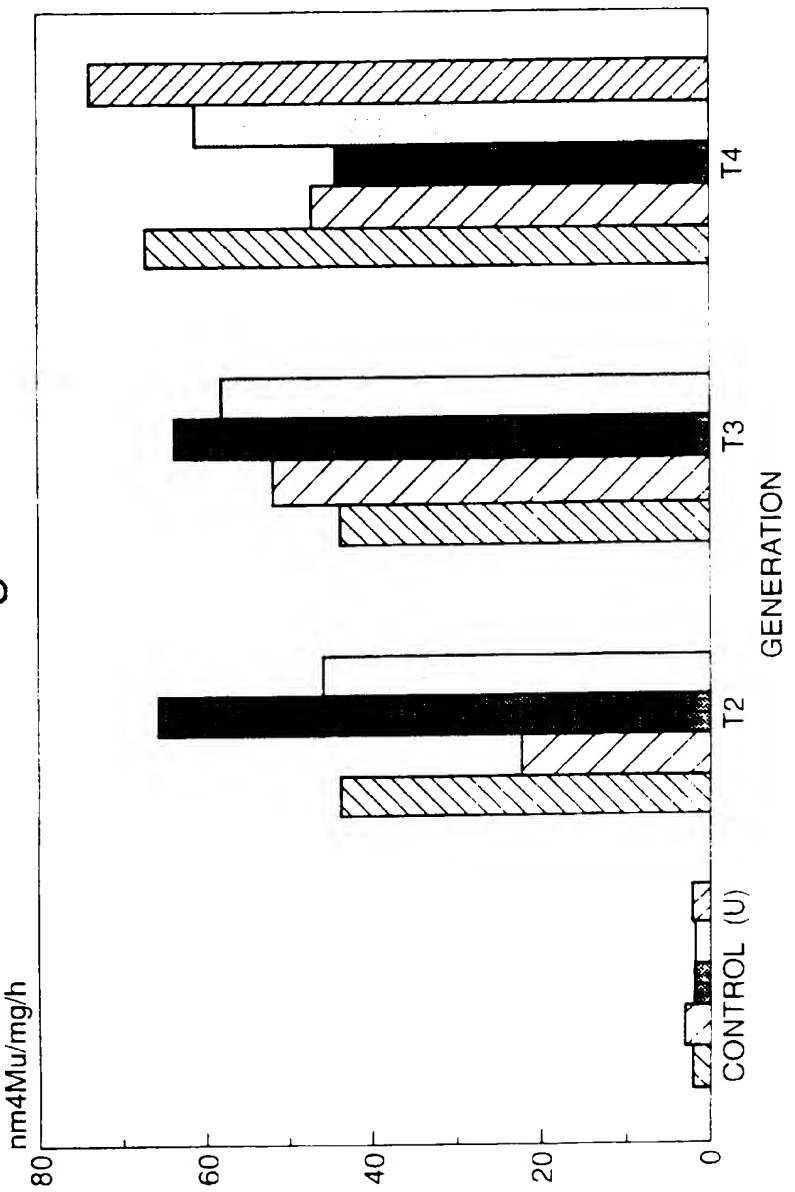


Fig.5.

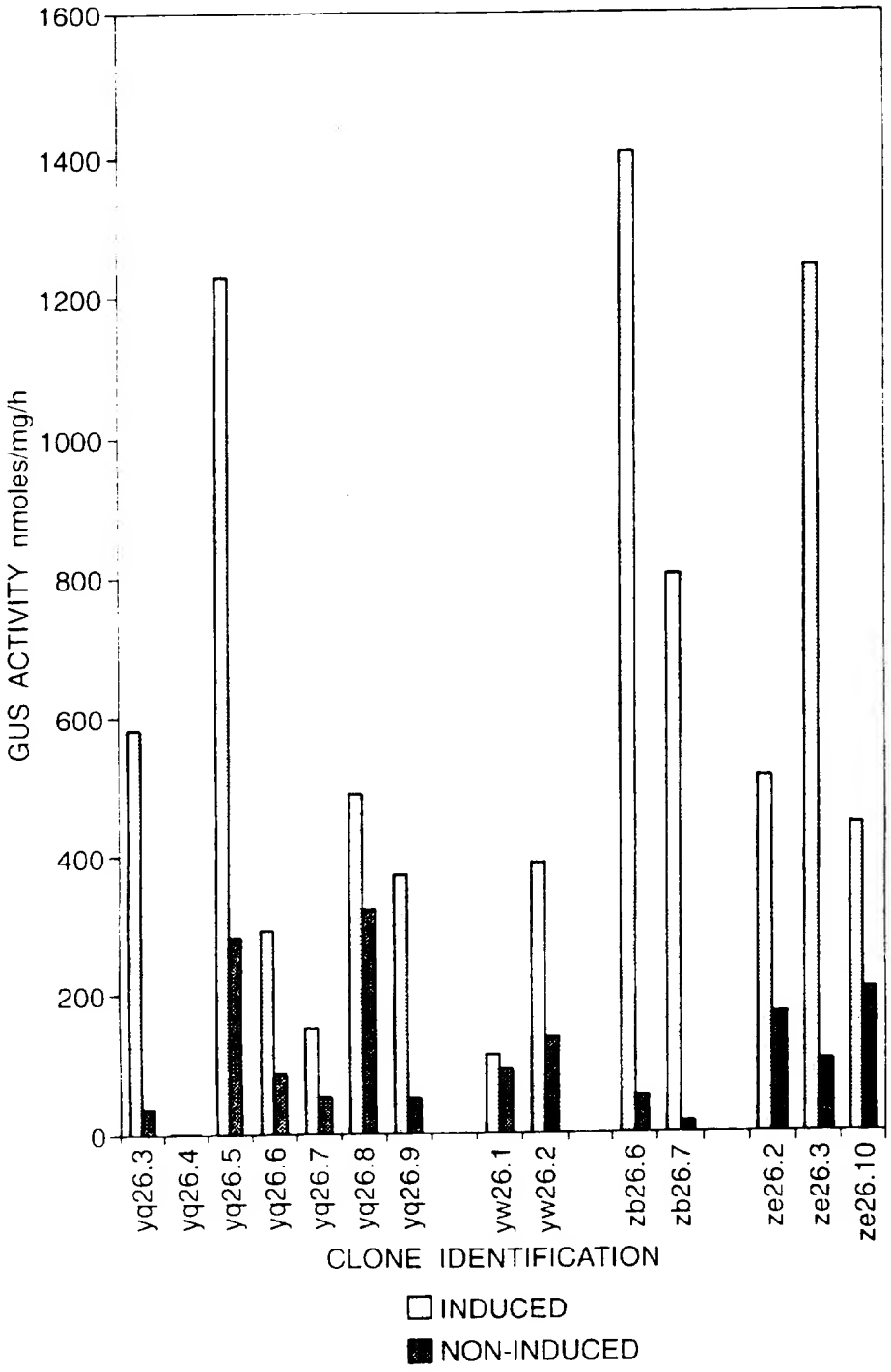

1600
1400
1200
1000
800
600
400
200
0
GUS ACTIVITY nmoles/mg/h
yq26.3
yq26.4
yq26.5
yq26.6
yq26.7
yq26.8
yq26.9
yw26.1
yw26.2
zb26.6
zb26.7
ze26.2
ze26.3
ze26.10
CLONE IDENTIFICATION
INDUCED
NON-INDUCED

Fig.6.

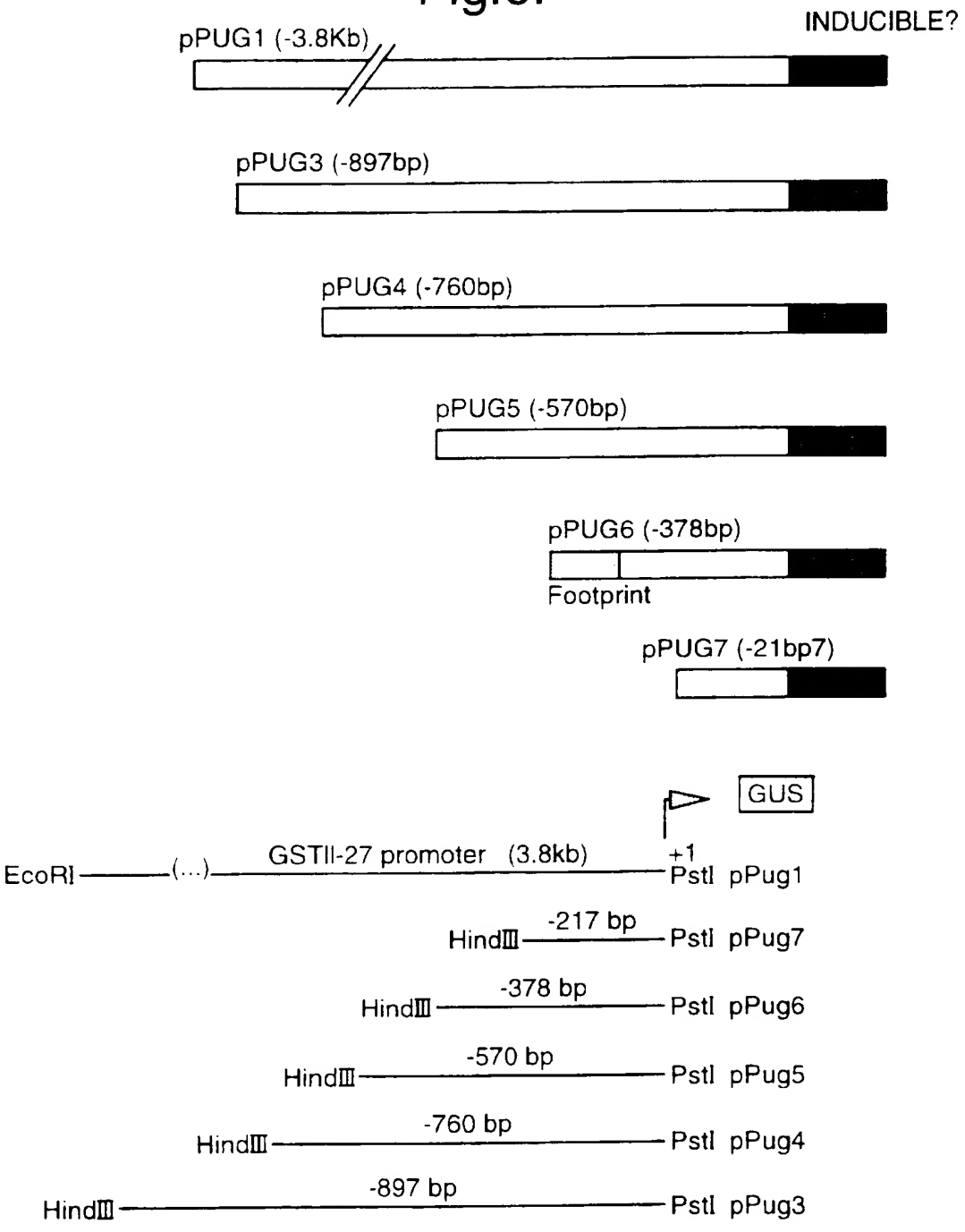

INDUCIBLE?
pPUG1 (-3.8Kb)
pPUG3 (-897bp)
pPUG4 (-760bp)
pPUG5 (-570bp)
pPUG6 (-378bp)
Footprint
pPUG7 (-21bp7)
GUS
EcoRI
(...)
GSTII-27 promoter (3.8kb)
+1
PstI pPug1
HindIII
-217 bp
PstI pPug7
HindIII
-378 bp
PstI pPug6
HindIII
-570 bp
PstI pPug5
HindIII
-760 bp
PstI pPug4
HindIII
-897 bp
PstI pPug3

Fig.7.

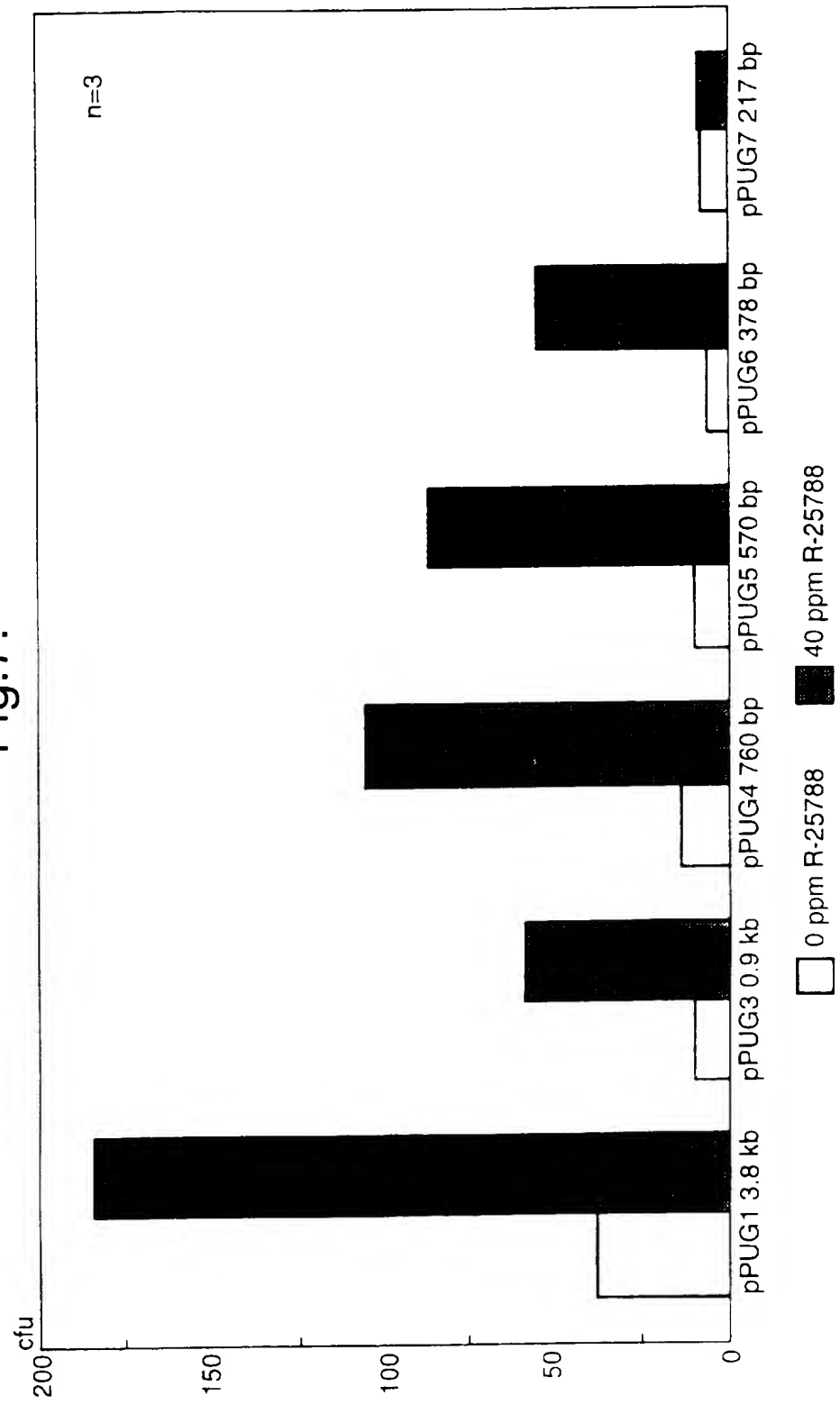
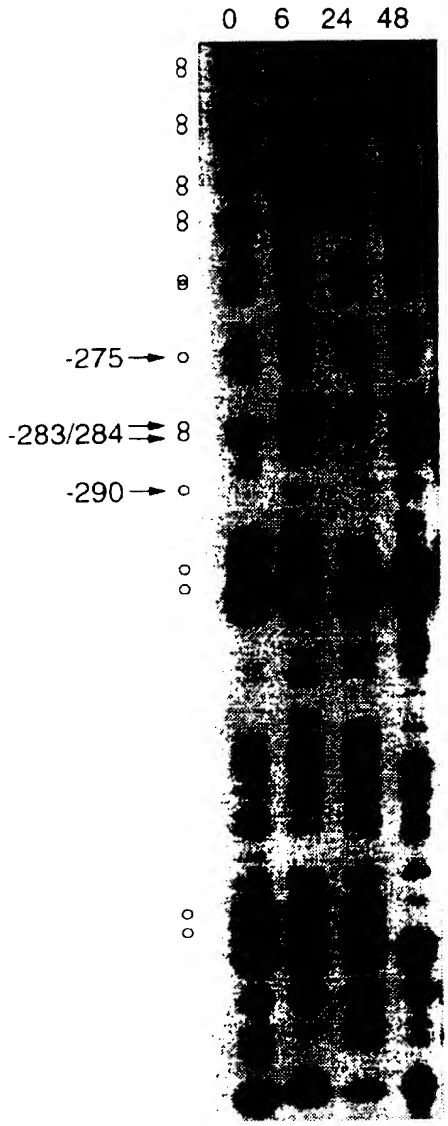

n=3
cfu
200
150
100
50
0
pPUG1 3.8 kb
pPUG3 0.9 kb
pPUG4 760 bp
pPUG5 570 bp
pPUG6 378 bp
pPUG7 217 bp
0 ppm R-25788
40 ppm R-25788

Fig.8.



GST-27 FOOTPRINT

-290 -284/283 -275

3' CAAGGATTTTAATAAAATTTTTAAAACCAAGAGTATAAAGTCTTAGGCTTTAAAGTATTTAGGTTTATCTGGCTTGG 5'

Fig.9.

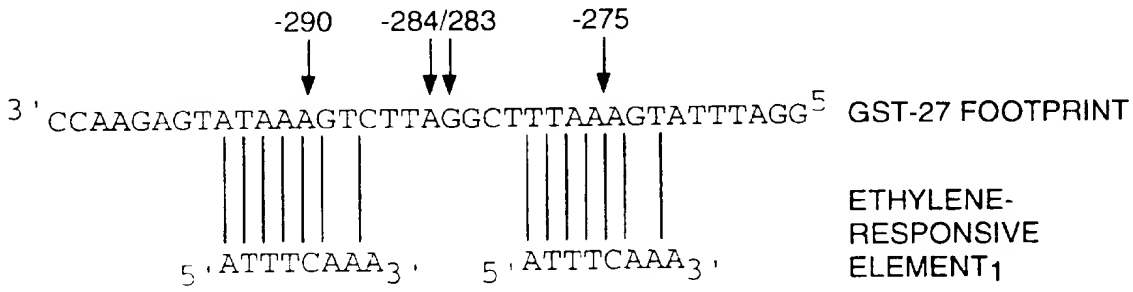

-290
-284/283
-275
3' CCAAGAGTATAAAGTCTTAGGCTTTAAAGTATTTAGG 5'
GST-27 FOOTPRINT
5' ATTTCAAA 3'
5' ATTTCAAA 3'
ETHYLENE-
RESPONSIVE
ELEMENT1

Fig.10.

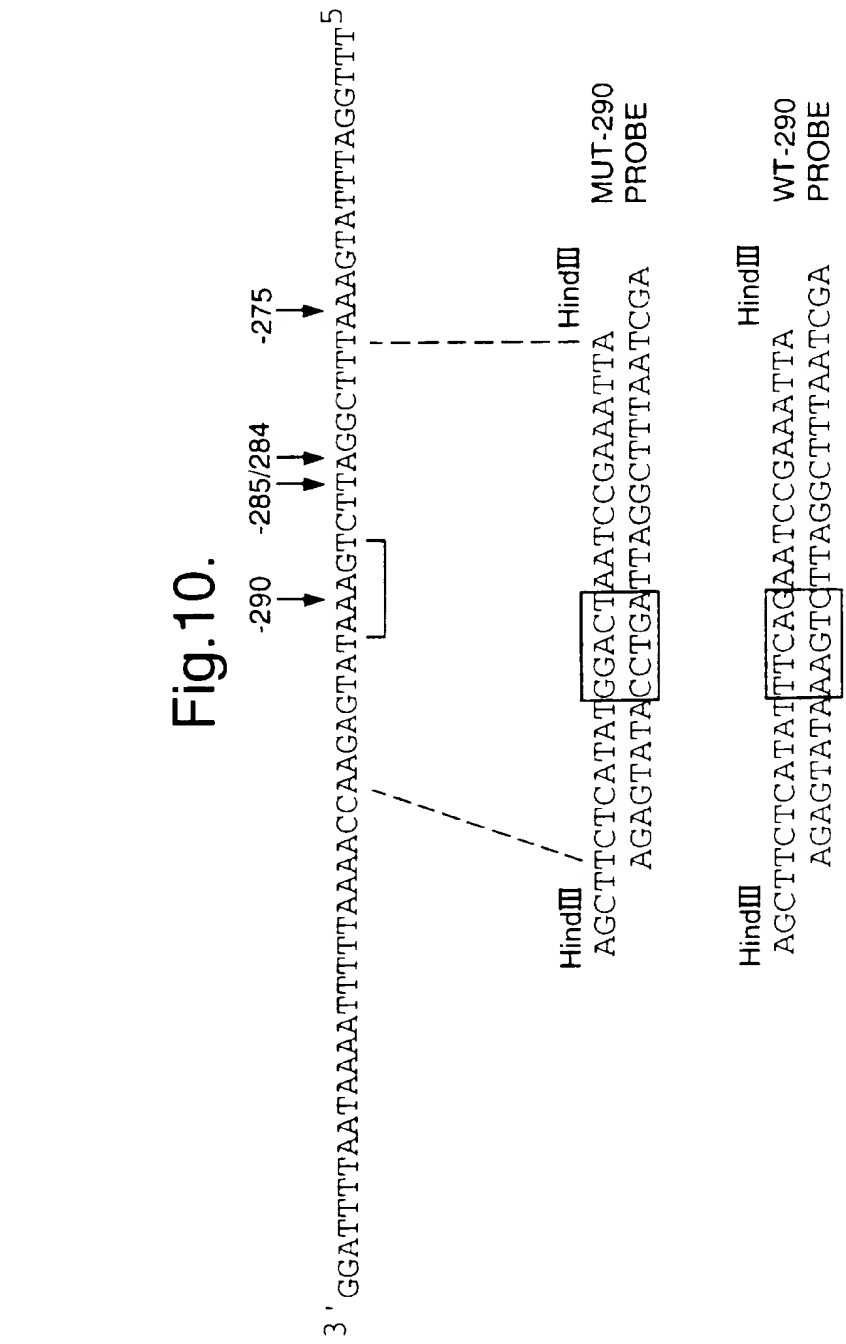

-290
-285/284
-275
3' GGATTTTAATAAAATTTTTAAAACCAAGAGTATAAAGTCTTAGGCTTTAAAGTATTTAGGTTT 5
HindIII
AGCTTCTCATATGGACTAATCCGAAATTA
AGAGTATACCTGATTAGGCTTTAATCGA
MUT-290 PROBE
HindIII
AGCTTCTCATATTTCAGAATCCGAAATTA
AGAGTATAAAGTCTTAGGCTTTAATCGA
WT-290 PROBE

Fig.11.

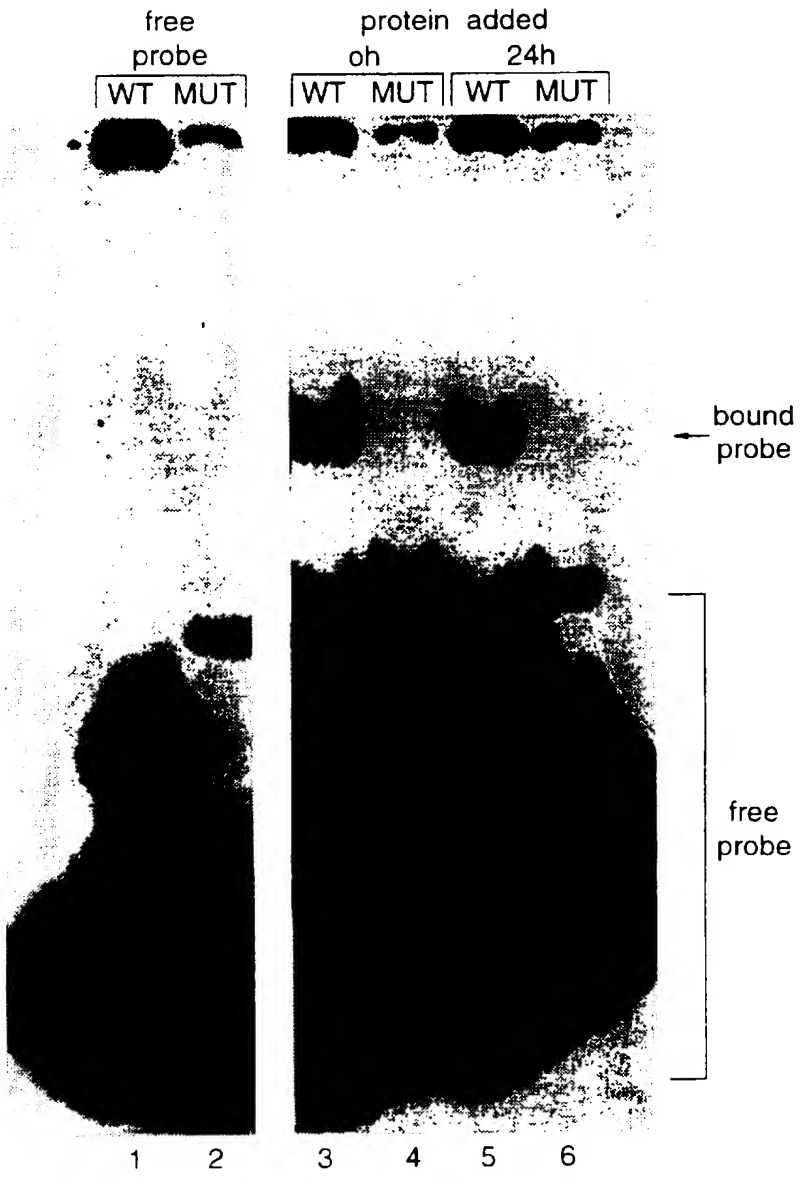

free probe
protein added
oh
24h
WT MUT
WT MUT
WT MUT
bound probe
free probe
1 2 3 4 5 6

Fig.12.

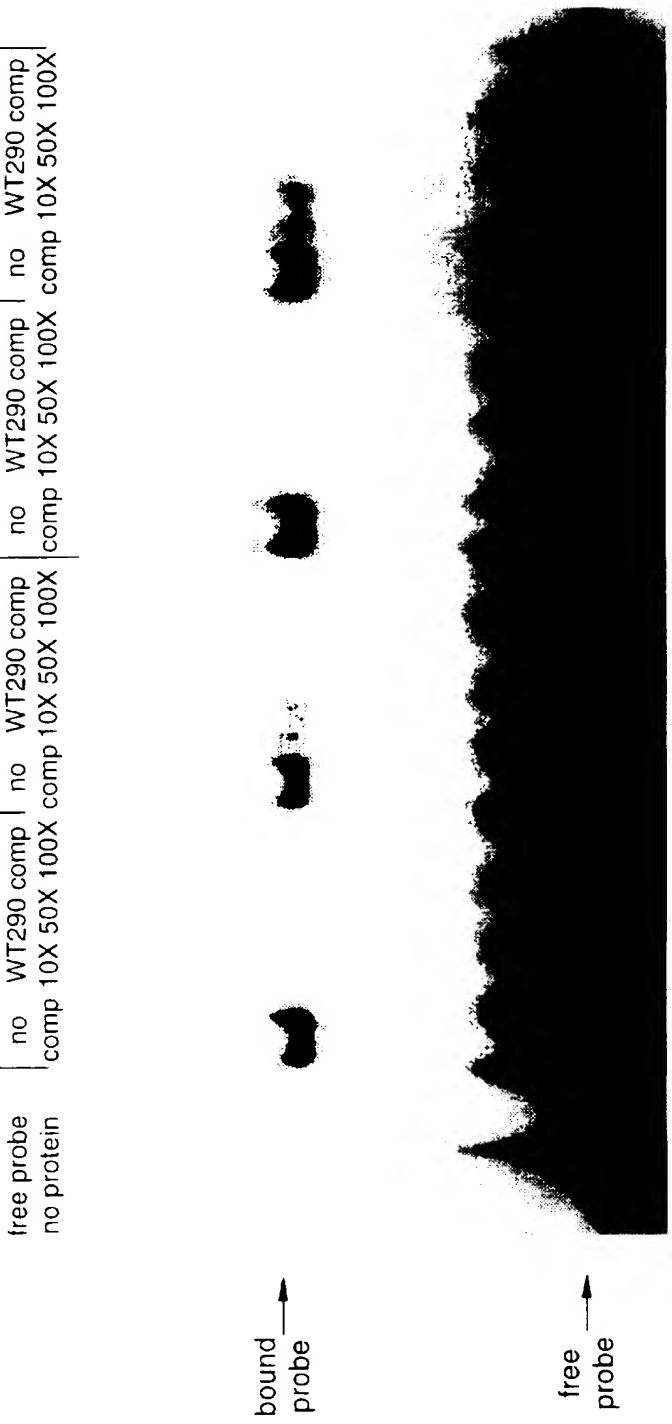

0h protein
24h protein
free probe
no protein
no comp
WT290 comp 10X
WT290 comp 50X
WT290 comp 100X
no comp
WT290 comp 10X
WT290 comp 50X
WT290 comp 100X
no comp
WT290 comp 10X
WT290 comp 50X
WT290 comp 100X
no comp
WT290 comp 10X
WT290 comp 50X
WT290 comp 100X
bound probe
free probe

Fig.13.

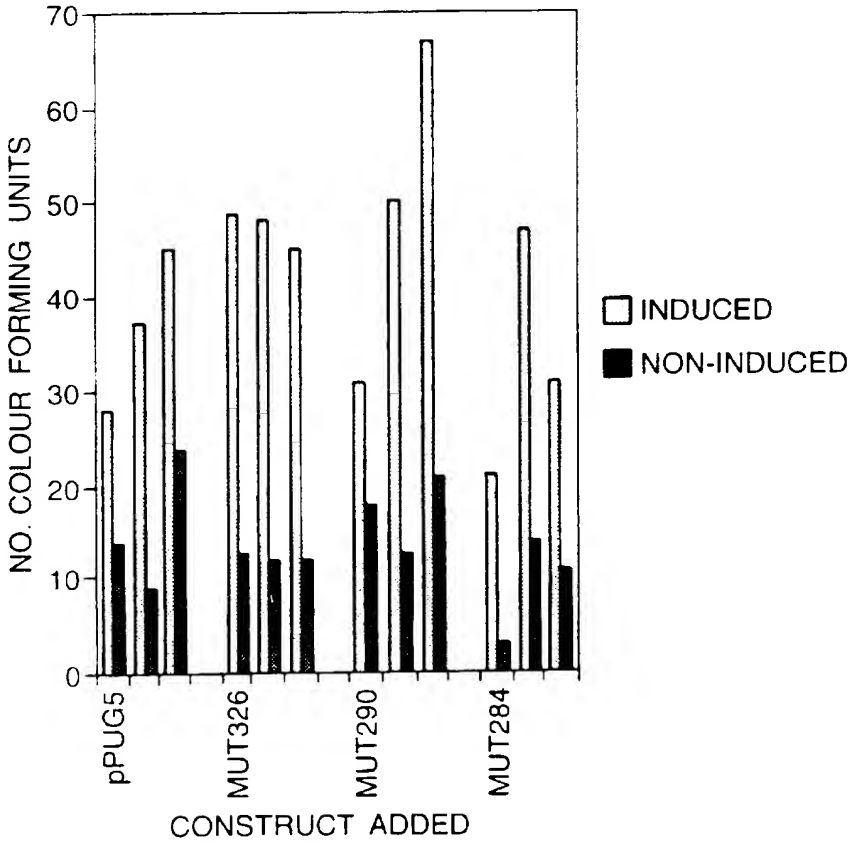
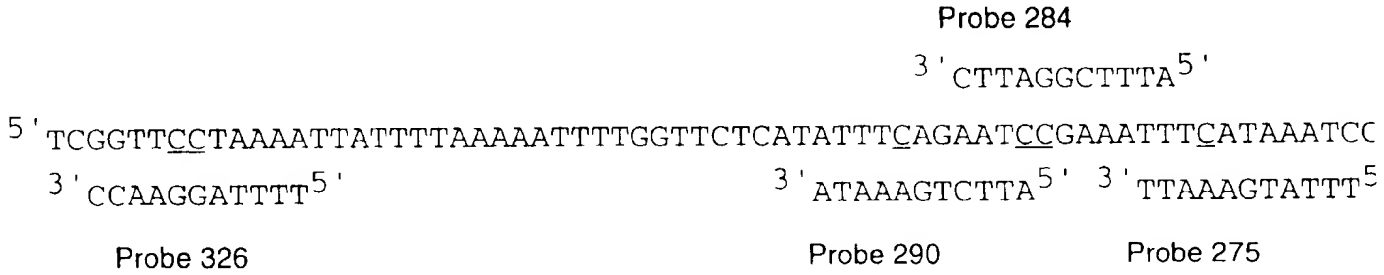

70
60
50
40
30
20
10
0
NO. COLOUR FORMING UNITS
INDUCED
NON-INDUCED
pPUG5
MUT326
MUT290
MUT284
CONSTRUCT ADDED

Fig.14.



Probe ERE TATTTCAAAAT
ATAAAGTTTTA

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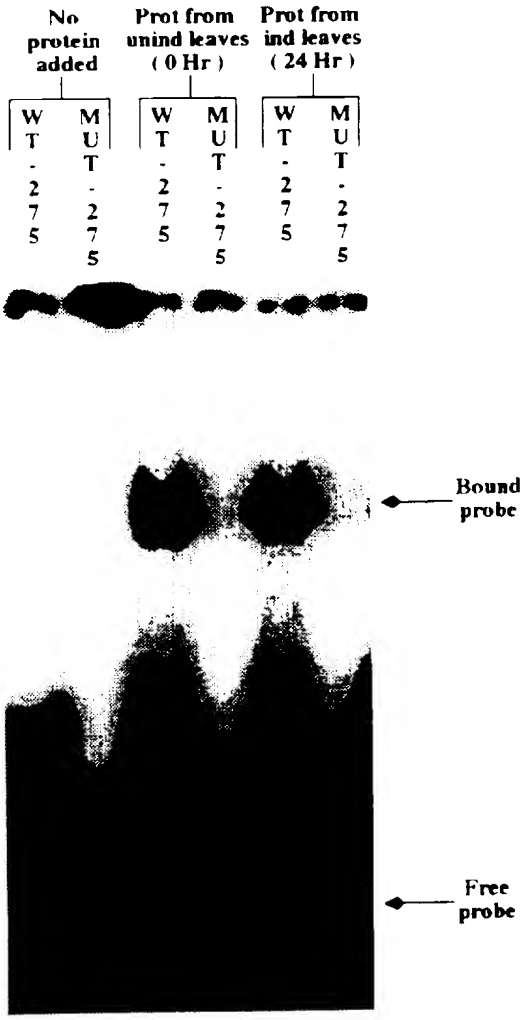
Fig.15.


No protein added
Prot from unind leaves (0 Hr)
Prot from ind leaves (24 Hr)
WT -275
MUT -275
WT -275
MUT -275
WT -275
MUT -275
Bound probe
Free probe

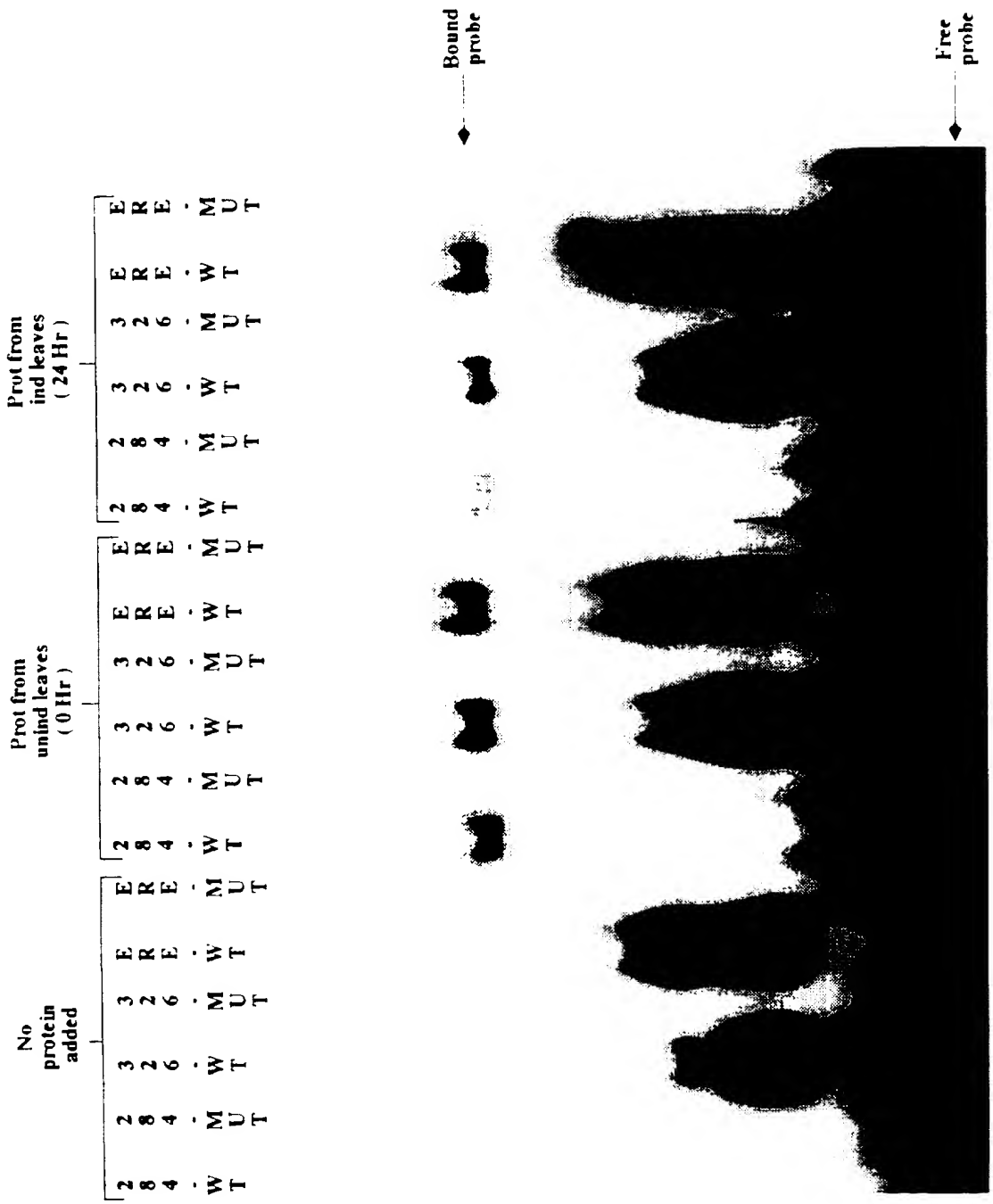
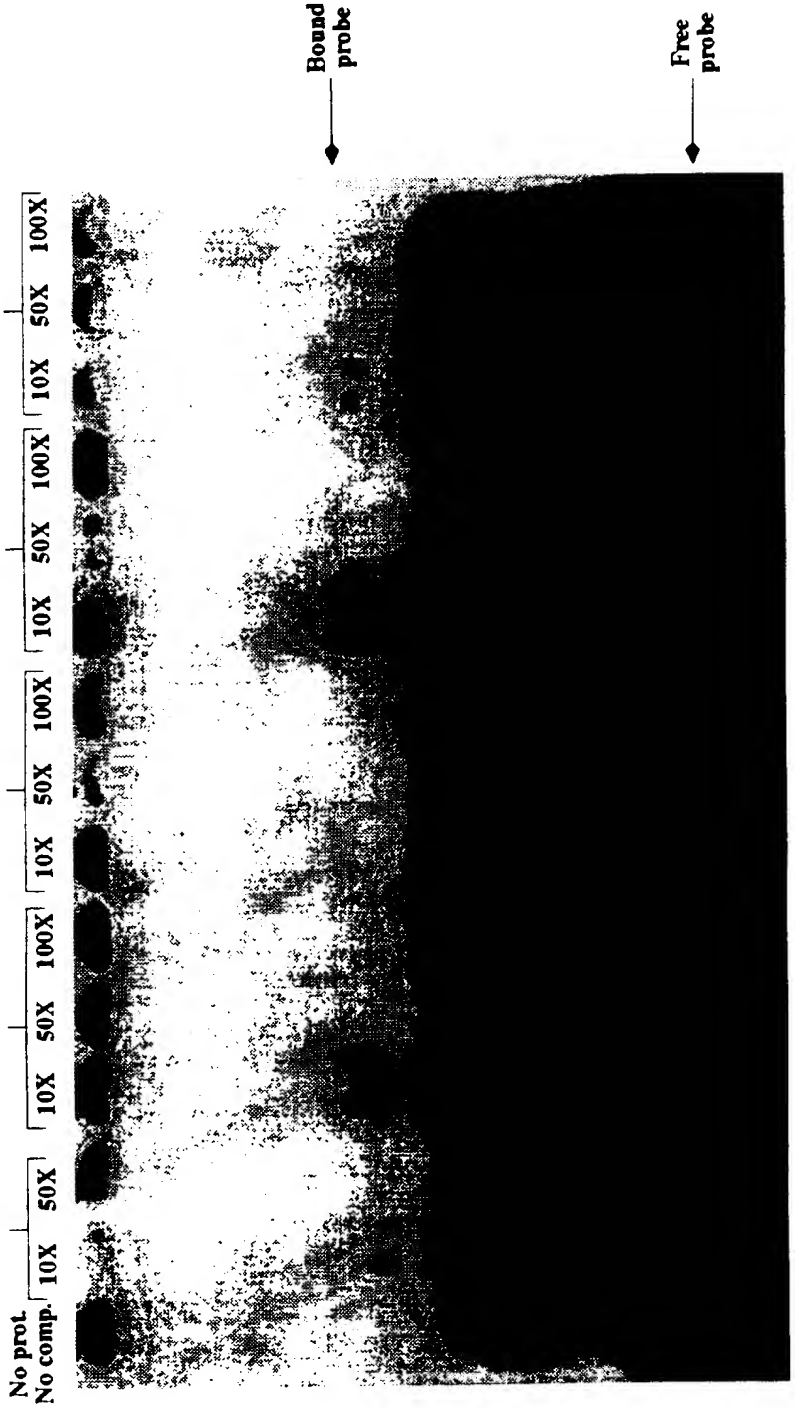

No protein added
Prot from unind leaves (0 Hr)
Prot from ind leaves (24 Hr)
Bound probe
Free probe
2 2 3 3 E E
8 8 2 2 R R
4 4 6 6 E E
- - - - - -
W M W M W M
T U T U T U
T T T T T T

Fig.16.

Fig.17.


Bound probe
Free probe
No prot.
No comp.
WT-290
10X
50X
WT-284
10X
50X
100X
WT-275
10X
50X
100X
WT-326
10X
50X
100X
WT-ERE
10X
50X
100X